

## Cyclic Adenosine Monophosphate Accumulation and $\beta$ -Adrenergic Binding in Unweighted and Denervated Rat Soleus Muscle

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Unweighting, but not denervation, of muscle reportedly "spares" insulin receptors, increasing insulin sensitivity. Unweighting also increases  $\beta$ -adrenergic responses of carbohydrate metabolism. These differential characteristics were studied further by comparing cyclic adenosine monophosphate (cAMP) accumulation and  $\beta$ -adrenergic binding in normal and 3-day unweighted or denervated soleus muscle. Submaximal amounts of isoproterenol, a  $\beta$ -agonist, increased cAMP accumulation in vitro and in vivo (by intramuscular [IM] injection) to a greater degree ( $P < .05$ ) in unweighted muscles. Forskolin or maximal isoproterenol had similar in vitro effects in all muscles, suggesting increased  $\beta$ -adrenergic sensitivity following unweighting. Increased sensitivity was confirmed by a greater receptor density ( $B_{max}$ ) for [ $^{125}$ I]iodo(-)-pindolol in particulate preparations of unweighted ( $420 \cdot 10^{-18}$  mol/mg muscle) than of control or denervated muscles ( $285 \cdot 10^{-18}$  mol/mg muscle). The three dissociation constant ( $K_d$ ) values were similar (20.3 to 25.8 pmol/L). Total binding capacity (11.4 fmol/muscle) did not change during 3 days of unweighting, but diminished by 30% with denervation. This result illustrates the "sparing" and loss of receptors, respectively, in these two atrophy models. In diabetic animals, IM injection of insulin diminished cAMP accumulation in the presence of theophylline in unweighted muscle ( $-66\% \pm 2\%$ ) more than in controls ( $-42\% \pm 6\%$ ,  $P < .001$ ). These results show that insulin affects cAMP formation in muscle, and support a greater in vivo insulin response following unweighting atrophy. These various data support a role for lysosomal proteolysis in denervation, but not in unweighting, atrophy.

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**P**REVIOUS STUDIES from this laboratory demonstrated enhanced insulin sensitivity of carbohydrate metabolism in rat soleus muscle unweighted (ie, weight-bearing function removed) for 3 to 6 days.<sup>1-4</sup> Increased insulin sensitivity was attributed to greater insulin-binding capacity per milligram of muscle in the unweighted soleus. In contrast, denervation produces insulin resistance of skeletal muscle carbohydrate metabolism,<sup>5-7</sup> associated with a postreceptor defect and no alteration in insulin-binding capacity.<sup>5,8</sup> Since membrane receptors may be degraded via the lysosomal pathway,<sup>9,10</sup> enhanced lysosomal proteolysis in denervated, but not unweighted, soleus could account for the contrasting effects of muscle atrophy on insulin-binding capacity in these two models.<sup>11</sup> Recently, we extended these previous studies to the  $\beta$ -adrenergic system, with the goal of linking enhanced  $\beta$ -adrenergic response to increased membrane-receptor binding. In parallel with heightened insulin sensitivity, there is a greater response of glycogen metabolism to isoproterenol, a  $\beta$ -agonist, in unweighted muscles compared with normal soleus muscles.<sup>4</sup> Therefore, in the current study, we investigated the mechanism of enhanced isoproterenol response in unweighted soleus by comparing cyclic adenosine monophosphate (cAMP) accumulation following receptor or postreceptor stimulation of normal and 3-day unweighted or denervated muscle. Further comparisons of the  $\beta$ -adrenergic systems in these muscles were made by measuring [ $^{125}$ I]iodo(-)-pindolol binding to particulate muscle preparations.

### MATERIALS AND METHODS

#### Treatment of Animals

Female Sprague-Dawley rats (60 to 75 g; Sasco, Omaha, NE) were maintained on food and water ad libitum. Suspended animals were tranquilized with Innovar-Vet (10  $\mu$ L/100 g body weight; Pitman-Moore, Mundelein, IL) and tail-casted as previously described.<sup>12</sup> Control animals remained weight-bearing and were neither tranquilized nor tail-casted, since neither of these manipulations alters  $\beta$ -adrenergic responses 3 days later. Bilateral dener-

vation of rat hindlimbs was performed under the combined administration of Innovar-Vet and ether. A small incision was made in the skin on the posterior aspect of the thigh, and the overlying muscle tissue was bluntly dissected to reveal the sciatic nerve. A small piece (2 to 3 mm) of the nerve was removed to prevent nerve regeneration, and the incision was closed with surgical wound clips. All treatments lasted 3 days.

To be made diabetic, overnight food-deprived rats were injected intraperitoneally (IP) with streptozotocin (85 mg/kg body weight, Sigma Chemical, St Louis, MO) in 0.9% saline. On the mornings of the third and fourth days after streptozotocin treatment, animals were injected subcutaneously (SC) with 5 U protamine zinc insulin (Eli Lilly, Indianapolis, IN) per 100 g body weight. For suspended animals, tail-casting was performed 2 hours after the last insulin injection, with the animals wrapped loosely in a towel to reduce stress. Insulin was then withdrawn from all animals so that 72 hours later the animals were diabetic, as estimated from blood glucose levels of at least 18 mmol/L. Glucose in deproteinized plasma was measured spectrophotometrically.<sup>13</sup>

#### cAMP Determinations

Animals were killed by cervical dislocation, and soleus muscles were excised and weighed. Muscles were preincubated for 30 minutes in 3 mL Krebs-Ringer bicarbonate solution (pH 7.4, 37°C) equilibrated with 95% O<sub>2</sub>:5% CO<sub>2</sub> and containing 5 mmol/L glucose, 5 mmol/L succinate, 4 mmol/L pyruvate, 4 mmol/L glutamate, 1.5% bovine serum albumin (fatty acid-free) and 10  $\mu$ U

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bovine insulin/mL. Muscles were then transferred for 10 minutes to fresh Krebs-Ringer bicarbonate buffer containing 5 mmol/L glucose, 25 mmol/L theophylline (to inhibit phosphodiesterase), 1.5% bovine serum albumin (fatty acid-free), and 10  $\mu$ U bovine insulin/mL with or without isoproterenol or forskolin (as indicated in the figures and tables). Insulin was included during incubations to duplicate conditions used in previous studies that demonstrated an increased isoproterenol response of glycogen metabolism in unweighted soleus muscle.<sup>4</sup>

Following incubation, muscles were blotted, frozen in liquid nitrogen, and homogenized in a Duall tube containing 0.5 mL acidic ethanol (1 mol/L HCl:ethanol, 1:100). Homogenates were transferred to Eppendorf tubes and centrifuged at  $12,000 \times g$  for 15 minutes. The supernatant solution was saved, and the pellet was washed with 0.5 mL ethanol:water (2:1) and centrifuged for an additional 10 minutes. Supernatants were combined and evaporated to dryness under a stream of nitrogen at 55°C. The residue was dissolved in 50 mmol/L TRIS and 4 mmol/L EDTA buffer (pH 7.5), and then frozen at -20°C until assayed. The volume of buffer (0.1 to 2.0 mL) for dissolving the residue was selected so that a 50- $\mu$ L aliquot of the sample would fall within the range of maximum sensitivity (0.5 to 4.0 pmol) for the cAMP assay. cAMP was assayed using a commercial protein-binding kit (Amersham, Arlington Heights, IL). Except for bovine insulin (Calbiochem, San Diego, CA), chemicals were obtained from Sigma.

#### Intramuscular Injections

Animals were injected intramuscularly (IM) as described previously<sup>11</sup> and adapted from Gerard et al.<sup>14</sup> Rats were tranquilized with Innovar-Vet. Both hindlimbs were shaved and the skin was swabbed with ethanol. An incision was made through the outside of the leg, and a curved blunt forceps was used to hook the soleus muscle. Then, 0.9% saline containing theophylline (62.5 mmol/L) was injected into the left muscle. The injection for the right muscle also included either isoproterenol (2.5  $\mu$ mol/L) or insulin (10 mU/mL). After 20 minutes, the muscles were excised and frozen in liquid nitrogen. Muscles were homogenized and cAMP content was determined as described above.

#### Hormone-Binding Study

Particulate preparations were obtained from muscles frozen in liquid nitrogen as described by Ligget et al.<sup>15</sup> Muscles were minced in ice-cold buffer (10 mmol/L TRIS, 5 mmol/L EDTA, pH 7.4) and homogenized in 20 volumes of the same buffer with a Polytron P10 (Brinkman Insts, Westbury, NY) at maximum speed for three 10-second bursts. Homogenates were filtered over nylon mesh (1 mm<sup>2</sup>) and centrifuged at  $37,000 \times g$  for 20 minutes at 4°C. The pellet was resuspended in the same buffer and washed twice using similar centrifugations. Final suspensions (~7 mg muscle wet weight/mL) were in incubation buffer (75 mmol/L TRIS, 25 mmol/L MgCl<sub>2</sub>, 5 mmol/L EDTA, pH 7.4).

For the binding assay, preparations (100  $\mu$ L) were incubated for 60 minutes at 25°C with [<sup>125</sup>I]iodo(-)-pindolol (2,200 Ci/mmol; New England Nuclear, Boston, MA) in a final volume of 150  $\mu$ L incubation buffer.<sup>15</sup> The reaction was terminated by adding 10 mL ice-cold incubation buffer and vacuum filtering through a Whatman GF/C glass fiber filter (Whatman International, Maidstone, England). Filters were washed with an additional 30 mL incubation buffer, and bound radioactivity was measured in a gamma-counter. Nonspecific binding was determined by linear regression of binding that occurred in the presence of 1  $\mu$ mol L-propranolol. Specific binding was calculated as the difference between total and nonspecific binding.

#### Data Analysis

Receptor densities ( $B_{max}$ ) and apparent dissociation constants ( $K_d$ ) were estimated by multiple iterative nonlinear analysis of saturation binding data using the computer program, LIGAND (Elsevier-Biosoft, Cambridge, UK).<sup>16</sup> Specific binding expressed per milligram muscle or per whole muscle was calculated using the total wet weight of tissue or total number of muscles represented by the 100- $\mu$ L particulate preparation used in the saturation binding experiments. Testing for significant differences between means ( $P < .05$ ) was done by a paired Student's *t* test or by factorial ANOVA with a post hoc Scheffé *F* test or Fisher exact probability test. Differences in percent effects of isoproterenol or insulin injections between groups were analyzed by the Mann-Whitney *U* test. All results are expressed as means  $\pm$  SE for the number of muscles indicated in each table or figure.

## RESULTS

#### Muscle and Body Masses

Weight-bearing (control) animals weighed approximately 15 g less initially than unweighted and denervated animals (Table 1), so that final soleus muscle mass would be more closely matched for incubations. Masses of muscles used for cAMP determinations were similar in weight-bearing and denervated muscles, while those from unweighted animals were slightly (6%) smaller. Similar final muscle size diminished the possibility of different diffusion distances. Unweighted animals gained less than weight-bearing or denervated animals. Since food consumption is similar in unweighted and weight-bearing animals,<sup>12</sup> this weight-gain difference is likely due to the mild stress effects associated with tail-cast suspension.<sup>17</sup> In both cAMP accumulation and hormone-binding experiments (not shown), the ratio of muscle to body mass, an index of muscle atrophy, was less in unweighted and denervated animals than in control animals.

#### cAMP Accumulation In Vitro

One potential mechanism for the greater effects of isoproterenol on glycogen metabolism in unweighted muscle<sup>4</sup> could be postreceptor alterations, such as in adenylate cyclase activity. Therefore, we measured accumulation of cAMP (in the presence of theophylline) following incubation with or without forskolin, which activates adenylate cyclase independent of the  $\beta$ -adrenergic receptor.<sup>18</sup> The only difference detected in basal cAMP accumulation was a lower ( $P < .05$ ) amount in unweighted than in denervated muscle (Table 2). Forskolin treatment increased cAMP

**Table 1. Muscle and Body Masses of Animals Used for cAMP Determinations**

Muscle	Body Mass (g)		Soleus Mass (mg)	Soleus:Body (mg:g)
	Initial	Final		
Normal	61.7 $\pm$ 1.0	76.2 $\pm$ 1.1	29.2 $\pm$ 0.4	0.383 $\pm$ .009
Unweighted	75.1 $\pm$ 1.1*	86.2 $\pm$ 1.1*†	28.1 $\pm$ 0.4*†	0.314 $\pm$ .005*
Denervated	76.2 $\pm$ 1.0*	91.6 $\pm$ 1.0*	28.6 $\pm$ 0.4	0.312 $\pm$ .004*

NOTE. Values are means  $\pm$  SE for 42 to 47 animals.

\* $P < .05$  unweighted or denervated versus weight-bearing by ANOVA.

† $P < .05$  unweighted versus denervated by ANOVA.

**Table 2. Effect of Forskolin on cAMP Accumulation in Vitro**

Forskolin (mmol/L)	Normal	Unweighted (pmol/muscle)	Denervated
0	8.6 ± 0.6	7.2 ± 0.5*	9.9 ± 0.6
0.1	81.4 ± 7.0†	81.6 ± 5.3†	73.0 ± 6.4†
0.3	137.0 ± 12.5†	138.3 ± 6.6†	136.7 ± 11.6†
0.5	169.4 ± 16.2†	137.8 ± 7.4*†	192.9 ± 10.9†
1.0	178.9 ± 1.8†	142.8 ± 7.0*†	215.2 ± 34.0†

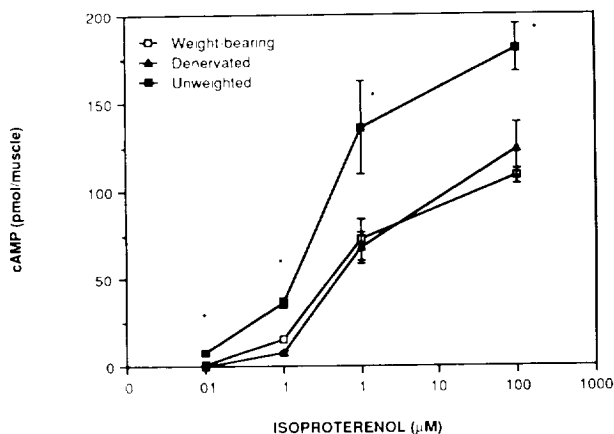
NOTE. Muscles were incubated as described in the Methods with 10  $\mu$ U insulin/mL and the forskolin concentration indicated. cAMP accumulation was also measured as described in the Methods. Values are means  $\pm$  SE for 4 to 19 muscles.

\* $P < .05$  unweighted versus denervated by ANOVA.

† $P < .05$  forskolin versus no forskolin by ANOVA.

accumulation in a dose-dependent manner in all conditions. A maximal effect was achieved at 0.5 mmol/L. Accumulation of cAMP in normal muscle did not differ from that in unweighted or denervated muscles at all forskolin concentrations tested. However, at the higher concentrations of forskolin, denervated muscle accumulated more cAMP than did unweighted muscle. These results suggest that the site of enhanced isoproterenol response in the unweighted soleus is likely proximal to the adenylate cyclase catalytic subunit in the  $\beta$ -adrenergic receptor-effector cascade.

Isoproterenol stimulated cAMP accumulation in a dose-dependent fashion in all three muscles (Fig 1). In accordance with an enhanced isoproterenol response of glycogen metabolism,<sup>4</sup> cAMP accumulation was markedly greater in unweighted than in weight-bearing or denervated muscles. Weight-bearing and denervated muscles showed similar responses. These differences in cAMP accumulation could not be attributed to variable muscle integrity, as cAMP in the medium was below the detectable (0.2 pmol) limit under all conditions. These data suggest an enhanced isoproterenol sensitivity of cAMP accumulation in unweighted relative to weight-bearing muscles.



**Fig 1. Effect of isoproterenol on cAMP accumulation.** Muscles were incubated as described in Methods with 10  $\mu$ U insulin/mL and the isoproterenol concentration indicated above. Values are means  $\pm$  SE for five to 12 muscles. \* $P < .05$  compared with weight-bearing.

### cAMP Accumulation In Vivo

To evaluate the  $\beta$ -adrenergic response of cAMP accumulation in vivo, soleus muscles were injected with 0.9% saline containing theophylline with or without isoproterenol (Table 3). In the absence of isoproterenol, cAMP accumulation was lower ( $-46\%$ ) in unweighted than in weight-bearing muscles. Injection of isoproterenol elevated muscle cAMP content in both conditions. In agreement with in vitro results, both the absolute and percent increases of cAMP accumulation were greater in unweighted compared with weight-bearing muscles.

A proportionately smaller solution volume had been injected into the unweighted muscles than into the weight-bearing muscles. Even so, it was possible that the differential responses, in part, were a result of a difference in isoproterenol concentration in the muscle. To estimate the approximate agonist amount present, we used the percent in vivo responses from Table 3 and the in vitro dose curve in Fig 1 to predict the approximate average isoproterenol concentration in these muscles. For both control and unweighted muscles, a concentration of 0.24  $\mu$ mol/L was estimated. Thus, the difference reported in Table 3 was characteristic of the muscles, and was not a consequence of some large difference in agonist concentration.

In vivo basal cAMP accumulation was significantly less in unweighted muscle (Table 3). Since the insulin sensitivity of unweighted soleus is increased<sup>1</sup> and insulin reportedly reduces cAMP levels in some tissues,<sup>19,20</sup> we tested in vivo whether insulin effects could account for the basal difference in cAMP accumulation. Thus, unweighted or weight-bearing muscles of diabetic animals were injected with theophylline with or without insulin (Table 4). Diabetic animals were used to abolish the potential influence of circulating insulin on muscle cAMP levels. Elimination of insulin abolished the difference in cAMP accumulation detected in the absence of isoproterenol (Table 3). Injection of insulin significantly reduced the accumulation of cAMP in both conditions, but produced an even greater

**Table 3. Effect of Isoproterenol on cAMP Accumulation In Vivo**

Muscle	Amount of cAMP (pmol/muscle)		Increase (%)
	No Isoproterenol	With Isoproterenol	
Weight-bearing	10.0 $\pm$ 1.7	40.9 $\pm$ 3.1*	309 $\pm$ 70
Unweighted	5.4 $\pm$ 0.5†	53.7 $\pm$ 4.1*†	894 $\pm$ 114‡

NOTE. Contralateral soleus muscles in eight weight-bearing or hindlimb-suspended animals were injected with theophylline with or without isoproterenol as described in the Methods. Weight-bearing muscles were injected with 4.0  $\mu$ L/100 g body weight, but unweighted muscles were injected with only 3.2  $\mu$ L/100 g body weight to account for muscle size differences owing to atrophy caused by unweighting. These volumes are based on average soleus muscle sizes of 40 and 32 mg/100 g body weight for weight-bearing and suspended animals, respectively. After 20 minutes, the muscles were excised and immediately processed for analysis of cAMP accumulation as described in the Methods. Results are means  $\pm$  SE.

\* $P < .001$  isoproterenol versus no isoproterenol by ANOVA or paired  $t$  test.

† $P < .05$  unweighted versus weight-bearing by ANOVA.

‡ $P < .001$  unweighted versus weight-bearing by Mann-Whitney.

**Table 4. Effect of Insulin on cAMP Accumulation In Vivo**

Muscle	Amount of cAMP (pmol/muscle)		Decrease (%)
	Without Insulin	With Insulin	
Weight-bearing	9.4 ± 0.8	5.3 ± 0.4*	41.5 ± 5.9
Unweighted	11.3 ± 1.2	3.8 ± 0.4*	65.8 ± 2.1†

NOTE. Muscles from diabetic rats were injected with theophylline with or without insulin, as in Table 3. Weight-bearing muscles were injected with 3.7  $\mu$ L/100 g body weight, and unweighted muscles were injected with 2.7  $\mu$ L/100 g body weight. Volumes are based on average soleus muscle sizes of 37 and 27 mg/100 g body weight for weight-bearing and suspended diabetic animals, respectively. Results are means  $\pm$  SE.

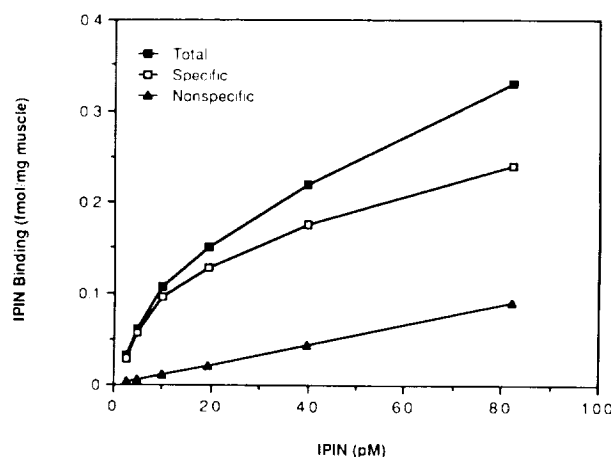
\* $P < .01$  insulin versus no insulin by ANOVA or paired  $t$  test.

† $P < .001$  unweighted versus weight-bearing by Mann-Whitney.

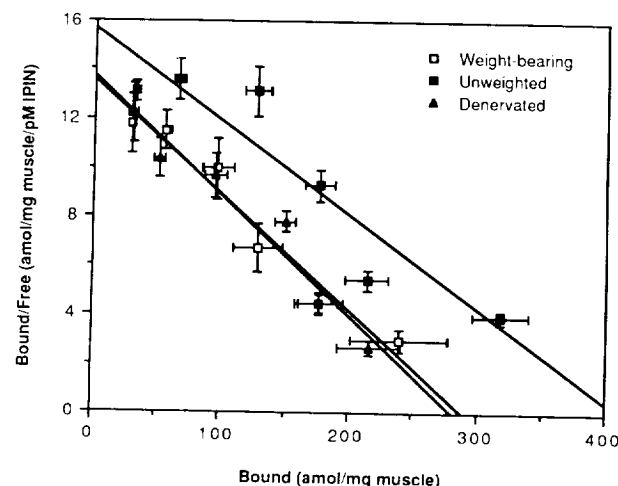
effect in the unweighted muscle, thereby restoring a significant difference, as between muscles of nondiabetic animals (see Tables 3 and 4).

### $\beta$ -Adrenergic Binding

To distinguish between altered hormone sensitivity and responsiveness, we measured the binding capacity of the membrane receptor. [ $^{125}$ I]iodo-(–)-pindolol saturation binding was measured with particulate preparations from weight-bearing, unweighted, and denervated muscles. Binding appeared saturable and could be inhibited by 1  $\mu$ M L-propranolol. Specific binding occurred in "zone A" (ie, <10% of total ligand bound) and represented between 70% to 90% of total maximal binding (Fig 2). Scatchard analysis<sup>21</sup> of binding data demonstrated similar receptor affinity ( $K_d$ ) for weight-bearing ( $20.7 \pm 1.9$  pmol/L), unweighted ( $25.8 \pm 3.3$  pmol/L), and denervated ( $20.3 \pm 2.1$  pmol/L) muscles (Fig 3). These values agree with the  $K_d$  (19.5 pmol/L) in [ $^{125}$ I]iodo-(–)-pindolol binding in human skeletal muscle.<sup>15</sup> Maximal binding capacity per mg muscle was markedly greater in the unweighted soleus compared with the weight-bearing and denervated muscles that yielded similar results (Fig 3). When expressed relative to the whole



**Fig 2.** [ $^{125}$ I]iodo-(–)-pindolol binding to weight-bearing soleus particulate preparations. Muscles were treated as described in Methods. Data points represent mean values from triplicate determinations in each of three separate experiments.



**Fig 3.** Scatchard analysis of [ $^{125}$ I]iodo-(–)-pindolol binding to particulate preparations from weight-bearing and 3-day unweighted or denervated muscles. Muscles were treated as described in Methods. Lines of best fit were computed by nonlinear regression as described in Methods. Each point represents the mean  $\pm$  SE of triplicate determinations from three or four separate experiments; amol =  $10^{-18}$  mol.

muscle, maximal binding capacity was lower in denervated ( $7.8 \pm 1.1$  fmol/muscle) than in weight-bearing ( $11.2 \pm 0.8$  fmol/muscle) or unweighted ( $11.5 \pm 1.2$  fmol/muscle) muscles. Similar general findings were obtained for binding of [ $^3$ H]dihydroalprenolol to membrane preparations from these muscles.<sup>22</sup> These results suggest that the increase in  $\beta$ -receptor number per mg muscle in unweighted soleus must be mostly a consequence of muscle atrophy and not of an increase in the total receptor population. The reduction in total  $\beta$ -receptors of denervated muscle suggests that the loss of this membrane protein parallels decreases in structural proteins.

## DISCUSSION

### Receptor and Postreceptor Stimulation of cAMP Accumulation

Hormone effects can be characterized by altered sensitivity or responsiveness representing receptor or postreceptor modifications, respectively.<sup>23</sup> Investigation herein of receptor and postreceptor stimulation of the  $\beta$ -adrenergic receptor-effector cascade supported our previous studies that suggested greater  $\beta$ -adrenergic sensitivity following unweighting.<sup>4</sup> Whether via incubation (Fig 1) or IM injection (Table 3), submaximal amounts of isoproterenol increased cAMP accumulation more so in unweighted muscle. Similar responses to maximal amounts of isoproterenol were in accordance with enhanced sensitivity.<sup>23</sup> This concept is further supported by the comparable postreceptor stimulation by forskolin of cAMP accumulation in unweighted and weight-bearing muscles (Table 2). Thus, increased responsiveness of adenylate cyclase cannot account for enhanced  $\beta$ -adrenergic effects in unweighted muscle.

These results cannot exclude enhanced G-protein-complex coupling between the receptor and adenylate

cyclase. For instance, decreased isoproterenol response of cardiac muscle from adrenalectomized animals occurs without altered  $\beta$ -receptor density or affinity.<sup>24</sup> Instead, the lack of glucocorticoids may alter  $\beta$ -adrenergic responses at a postreceptor site. As dexamethasone treatment reverses reductions in the  $G_\beta$ -protein subunit mRNA of adipocytes from adrenalectomized rats and increases this mRNA in normal animals, glucocorticoids may modulate  $\beta$ -adrenergic receptor-effector coupling.<sup>25</sup> The several-fold increases of plasma glucocorticoids<sup>17</sup> and soleus glucocorticoid receptors<sup>26</sup> following unweighting could possibly alter the  $\beta$ -adrenergic receptor-effector cascade. Further studies are needed to evaluate this possibility.

A possible role of systemic effects in altered hormone responses of unweighted versus weight-bearing muscles has been evaluated by examining insulin and isoproterenol responses of the extensor digitorum longus, a hindlimb muscle unaffected by hindlimb unweighting. Insulin stimulation of glucose transport<sup>1,3</sup> and isoproterenol stimulation of lactate production<sup>4</sup> were similar in this muscle from control and suspended animals. Therefore, differences in hormone responses between weight-bearing and unweighted soleus muscles are not likely due to a systemic alteration.

In our earlier study,<sup>4</sup> incubations contained physiological amounts of insulin to assess insulin antagonism of isoproterenol effects in unweighted or denervated muscles. The insulin resistance of carbohydrate metabolism in denervated muscle<sup>7,8</sup> prevented us from evaluating this question. Results from the current study clearly indicate that neither receptor- nor postreceptor-mediated stimulation of cAMP accumulation is altered 3 days after denervating the soleus (Table 2 and Fig 1). These results agree with the similar forskolin-stimulated adenylate cyclase activity and [<sup>3</sup>H]forskolin binding in 10-day denervated gastrocnemius.<sup>27</sup> In contrast, a 50% decline in basal and catecholamine- or fluoride-stimulated adenylate cyclase activity occurred for a mixture of hindlimb muscles denervated for 5 days.<sup>28</sup> This decrease was attributed to reduced amounts of adenylate cyclase enzyme per muscle. The reason for the discrepancy between these two studies is unclear. Possibly, the absence of phosphodiesterase inhibitors in one study<sup>28</sup> confounds the specific determination of differences in adenylate cyclase activity.

#### *Insulin Effects on cAMP Accumulation*

Previous studies reported increased effects of insulin on carbohydrate and protein metabolism in unweighted muscle.<sup>1,7,29</sup> Additionally, the greater insulin sensitivity of unweighted muscle leads to a lower accumulation of cAMP (Table 4). To our knowledge, this is the first investigation to demonstrate a reduced accumulation of cAMP in skeletal muscle following insulin treatment.

Several investigations have demonstrated an increased production of intracellular insulin mediators following insulin treatment of skeletal muscle.<sup>30-32</sup> These mediators increase the activity of low-K<sub>m</sub> phosphodiesterase and decrease adenylate cyclase activity in adipocyte and hepatocyte membranes.<sup>32-34</sup> However, neither in vitro, in vivo, nor

in situ insulin treatment diminished skeletal muscle cAMP levels in previous studies.<sup>35,36</sup> In this investigation, an effect of intracellular insulin mediators on phosphodiesterase activity was unlikely, as theophylline was always present. However, these results are consistent with insulin antagonism of isoproterenol-stimulated lactate formation in muscle.<sup>4,37</sup> While these findings support a response of cAMP metabolism to insulin in muscle, they cannot exclude possible insulin effects at sites other than adenylate cyclase. For example, insulin inhibition of cAMP-dependent protein kinase activity<sup>38</sup> could also explain the diminished formation of lactate.

The inability of previous investigators to detect insulin effects on muscle cAMP metabolism could be due to the absence of phosphodiesterase inhibitors in those studies.<sup>35,36</sup> Furthermore, the use of muscles from diabetic animals may allow detection of small differences in cAMP accumulation due to insulin. While the physiologic significance of these responses remains to be determined, small changes in skeletal muscle cAMP levels by insulin could possibly result in large changes in cellular metabolism through amplification.

#### *$\beta$ -Adrenergic Binding Capacity in Atrophic Soleus*

In accordance with the concept that altered hormone sensitivity is a receptor-mediated phenomenon,<sup>23</sup>  $\beta$ -adrenergic binding capacity increased during unweighting atrophy (Fig 3). Just as increased effects of insulin paralleled greater insulin binding capacity in unweighted soleus,<sup>1,29</sup> enhanced isoproterenol effects in unweighted muscle can be attributed to increased  $\beta$ -adrenergic-receptor concentration. Mechanisms for increased  $\beta$ -receptor density may include: (1) up-regulation due to reduced circulating catecholamines<sup>39</sup>; (2) changes in plasma glucocorticoids, which induce  $\beta$ -receptor expression<sup>40</sup>; or (3) sparing of membrane receptors during unweighting atrophy.<sup>1</sup> Catecholamine-induced up-regulation of the  $\beta$ -receptor seems unlikely, as plasma catecholamines increase during the first several days of suspension.<sup>41</sup> As we did not detect an increase in the total receptor population, it is not likely that increased plasma glucocorticoids<sup>17</sup> induced  $\beta$ -receptor expression during unweighting. However, these data cannot exclude a role for glucocorticoids in maintaining  $\beta$ -receptors during unweighting atrophy. Just as for insulin receptors,<sup>1</sup> there are a similar number of  $\beta$ -receptors per whole unweighted or weight-bearing muscle. Thus, the increase in receptor density per mg muscle must result from preferential loss of structural proteins rather than from up-regulation. It is noteworthy that the percent increase in  $\beta$ -adrenergic receptors (46%) (Fig 3) and insulin receptors (50%)<sup>1</sup> agree.

Isoproterenol responses of cAMP accumulation (Fig 1) and [<sup>125</sup>I]iodo-(+)-pindolol binding capacity per milligram muscle (Fig 3) were similar in denervated and innervated soleus muscles. The reduced total binding capacity (fmol/muscle) following denervation suggests that receptor and nonreceptor proteins are lost proportionately, thus preserving a receptor density comparable to innervated muscle. This constancy of  $\beta$ -adrenergic binding capacity was also evident in a mixed hindlimb muscle membrane preparation

following 5 days of denervation.<sup>28</sup> This similar  $\beta$ -adrenergic binding capacity in innervated and denervated muscles parallels their similar insulin binding capacity.<sup>5</sup> These results support the concept that, even though both unweighted and denervated muscles undergo atrophy, certain hormone responses and receptor binding capacities differ distinctly in these models of reduced use.

#### *Mechanisms of Proteolysis in Unweighted and Denervated Soleus*

A principal goal of studies from our laboratory has been to evaluate the possibility of different mechanisms of

proteolysis in unweighted and denervated soleus muscles.<sup>11</sup> Recent evidence suggests that membrane receptors may be degraded primarily through lysosomal proteolysis.<sup>9,10</sup> Thus, sparing of insulin receptors in unweighted,<sup>1</sup> but not in denervated,<sup>8</sup> muscle supports the idea that lysosomal proteolysis plays a greater role in denervation than in unweighting atrophy. Accordingly, IM injection of chloroquine, a lysosomotropic agent, diminished atrophy and in vivo proteolysis of the denervated, but not of the unweighted, soleus muscle.<sup>11</sup> The finding here of increased  $\beta$ -adrenergic receptor density with unweighting, but not with denervation, atrophy also supports this hypothesis.

#### REFERENCES

- Henriksen EJ, Tischler ME, Johnson DG: Increased response to insulin of glucose metabolism in the 6-day unloaded rat soleus muscle. *J Biol Chem* 261:10707-10712, 1986
- Henriksen EJ, Tischler ME: Glucose uptake in rat soleus: Effect of acute unloading and subsequent reloading. *J Appl Physiol* 64:1428-1432, 1988
- Henriksen EJ, Tischler ME: Time course of the response of carbohydrate metabolism to unloading in the soleus. *Metabolism* 37:201-208, 1988
- Kirby CR, Tischler ME:  $\beta$ -Adrenergic effects on carbohydrate metabolism in the unweighted rat soleus muscle. *J Appl Physiol* 69:2113-2119, 1990
- Smith RL, Lawrence JC: Insulin action in denervated skeletal muscle. Evidence that the reduced stimulation of glycogen synthesis does not involve decreased insulin binding. *J Biol Chem* 260:273-278, 1985
- Burant CF, Treutelaar MK, Buse MG: In vitro and in vivo activation of the insulin receptor kinase in control and denervated skeletal muscle. *J Biol Chem* 261:8985-8993, 1986
- Tischler ME, Satarug S, Eisenfeld SH, et al: Insulin effects in denervated and non-weight bearing rat soleus muscle. *Muscle Nerve* 13:593-600, 1990
- Burant CF, Lemmon SK, Treutelaar MK, et al: Insulin resistance of denervated rat muscle: A model for impaired receptor-function coupling. *Am J Physiol* 247:E657-E666, 1984
- Hare JF: Dissection of membrane protein degradation mechanisms by reversible inhibitors. *J Biol Chem* 263:8759-8764, 1988
- Hare JF: Mechanisms of membrane protein turnover. *Biochim Biophys Acta* 1031:71-90, 1990
- Tischler ME, Rosenberg S, Satarug S, et al: Different mechanisms of increased proteolysis in atrophy induced by denervation or unweighting of rat soleus muscle. *Metabolism* 39:756-763, 1990
- Jaspers SR, Tischler ME: Atrophy and growth failure of rat hindlimb muscles in tail-cast suspension. *J Appl Physiol* 57:1472-1479, 1984
- Bergmeyer HU, Bernt E, Schmidt F, et al: D-Glucose, in Bergmeyer HU (ed): *Methods of Enzymatic Analysis*, (vol 3). Deerfield Beach, FL, Verlag Chemie International, 1981, pp 1196-1201
- Gerard KW, Jacobs JW, Schneider DL: Rate of degradation of muscle proteins following localized radiolabeling with *N*-ethylmaleimide in vivo. *Arch Biochem Biophys* 181:94-102, 1977
- Liggett SB, Shah SD, Cryer PE: Characterization of  $\beta$ -adrenergic receptors of human skeletal muscle obtained by needle biopsy. *Am J Physiol* 254:E795-E798, 1988
- Munson PJ, Rodbard D: LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* 107:220-239, 1980
- Jaspers SR, Henriksen EJ, Satarug S, et al: Effects of stretching and disuse on amino acids in muscles of rat hind limbs. *Metabolism* 38:303-310, 1989
- Daly JW: Forskolin, adenylate cyclase, and cell physiology: An overview, in Greengard P, Robison GA, Paoletti R, et al (eds): *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*, vol 17. New York, NY, Raven, 1984, pp 81-99
- Butcher RW, Sneyd JGT, Park CR, et al: Effect of insulin on adenosine 3',5'-monophosphate in the rat epididymal fat pad. *J Biol Chem* 241:1651-1653, 1966
- Robison GA, Butcher RW, Sutherland EW: *Cyclic AMP*. New York, NY, Academic, 1971, pp 271-284
- Scatchard G: The attraction of proteins for small molecules and ions. *Ann NY Acad Sci* 51:560-572, 1949
- Kirby CR: Carbohydrate metabolism and the  $\beta$ -adrenergic system in disuse muscle atrophy. PhD Dissertation, University of Arizona, Tucson, 1990
- Kahn CR: Insulin resistance, insulin insensitivity, and insulin unresponsiveness: A necessary distinction. *Metabolism* 27:1893-1902, 1978
- Davies AO, De Lean A, Lefkowitz RJ: Myocardial  $\beta$ -adrenergic receptors from adrenalectomized rats: Impaired formation of high-affinity agonist-receptor complexes. *Endocrinology* 108:720-722, 1981
- Ros M, Watkins DC, Rapiejko PJ, et al: Glucocorticoids modulate mRNA levels for G-protein  $\beta$ -subunits. *Biochem J* 260:271-275, 1989
- Steffen JM, Musacchia XJ: Disuse atrophy, plasma corticosterone, and muscle glucocorticoid receptor levels. *Aviat Space Environ Med* 58:996-1000, 1987
- Hashimoto K, Watanabe Y, Uchida S, et al: Increase in the amount of adenylate cyclase in rat gastrocnemius muscle after denervation. *Life Sci* 44:1887-1895, 1989
- Smith PB, Greffrath SP, Appel SH:  $\beta$ -Adrenergic receptor-adenylate cyclase of denervated sarcolemmal membrane. *Exp Neurol* 59:361-371, 1978
- Bonen A, Elder GCB, Tan MH: Hindlimb suspension increases insulin binding and glucose metabolism. *J Appl Physiol* 65:1833-1839, 1988
- Larner J, Galasko J, Cheng K, et al: Pyruvate dehydrogenase activation in adipocyte mitochondria by an insulin-generated mediator from muscle. *Science* 206:1407-1408, 1979
- Jarett L, Wong EH, Smith JA, et al: Stimulation of pyruvate dehydrogenase activity in intact rat adipocytes by insulin mediator from rat skeletal muscle. *Endocrinology* 116:1011-1016, 1985
- Macaulay SL, Jarett L: Insulin mediator causes dephosphorylation of the  $\alpha$ -subunit of pyruvate dehydrogenase by stimulating phosphatase activity. *Arch Biochem Biophys* 237:142-150, 1985
- Jarett L, Kiechle FL, Macaulay SL, et al: Intracellular

mediators of insulin action, in Czech MP (ed): *Molecular Basis of Insulin Action*. New York, NY, Plenum, 1985, pp 183-198

34. Cheng K, Thompson M, Schwartz C, et al: Multiple intracellular peptide mediators of insulin action, in Czech MP (ed): *Molecular Basis of Insulin Action*. New York, NY, Plenum, 1985, pp 171-182

35. Craig JW, Rall TW, Larner J: The influence of insulin and epinephrine on adenosine 3',5'-phosphate and glycogen transferase in muscle. *Biochim Biophys Acta* 177:213-219, 1969

36. Bauer HP, Birkel G, Hofer HW: The concentrations of glucose 1,6 bisphosphate and other regulatory metabolites, and the activities of enzymes of the glycogen metabolism in the perfused rabbit psoas muscle. *Int J Biochem* 18:73-77, 1986

37. Challiss RAJ, Lozeman FJ, Leighton B, et al: Effects of the

$\beta$ -adrenoceptor agonist isoprenaline on insulin-sensitivity in soleus muscle of the rat. *Biochem J* 233:377-381, 1986

38. Kida Y, Nyomba BL, Bogardus C, et al: Defective insulin response of cyclic adenosine monophosphate-dependent protein kinase in insulin-resistant humans. *J Clin Invest* 87:673-679, 1991

39. Stiles GL, Caron MG, Lefkowitz RJ:  $\beta$ -Adrenergic receptors: Biochemical mechanisms of physiological regulation. *Physiol Rev* 64:661-743, 1984

40. Davies AO, Lefkowitz RJ: Regulation of  $\beta$ -adrenergic receptors by steroid hormones. *Annu Rev Physiol* 46:119-130, 1984

41. Woodman CR, Stump CS, Stump JA, et al: Influences of chemical sympathectomy and simulated weightlessness on male and female rats. *J Appl Physiol* 71:1005-1014, 1991

